Example 3

Recombinant Modified Vaccinia Ankara (MVA) Expressing the TAA lac Z and IC-2

Modified Vaccinia Ankara (MVA) strain is used as the expression vector. MVA was derived from vaccinia virus (WT) by over 570 serial passages in chicken embryo fibroblast cells (CEF) (Mayr, A. et al. Infection 3:6-14, 1975). The resulting MVA strain lost the capacity to productively infect mammalian cells (Altenburger, W. et al. Arch. Virol 105:15-27, 1989; Meyer, H. et al. J. Gen. Virol 72:1031-1038, 1991). The expression of late, as well as early, viral genes is unimpaired in human cells despite the inability of MVA to produce infectious progeny (Sutter and Moss, Proc. Natl. Acad. Sci. USA 89:10847-10851, 1992).

An insertion plasmid is constructed with the lac Z gene and the B7.1 gene under the control of the vaccinia virus late promotor P11 to allow homologous recombination at the site of a naturally occurring 3500-base-pair deletion within the MVA genome. MVA recombinants are isolated and propagated in permissive avian cells as described in Sutter and Moss 1992 and Sutter et al. Virology 1994. The expression of β-gal and B7.1 upon infection of nonpermissive human cells is detected by methods described herein.

Example 4

Effect Of Inoculation Of Various Recombinant Vaccinia Virus Constructs On Primary And Secondary Responses Against Vaccinia Or β-Galactosidase

Materials and Methods

5 BALB/c (H-2^d) mice per group were inoculated i.v. or s.c. with different doses the following vaccinia recombinant (rVV): IL-2 rVV; GM-CSF-rVV; TNFα-rVV; IFNγ-rVV.

In all these constructs the mouse model tumor

associated antigen, β-Gal was under the control of the p7.5-kD (E/L) promoter (Cahran, M.A. et al J. Virol. 54 (No. 1):30-37, 1995) while the cytokine production was driven by $p_{synthetic\ late}$ promoter (Davidson et al Nucleic Acid Research 18 (No. 14):4285-5286, 1991). All these rVV were generated by recombination in the TK region of vaccinia using the plasmid pMJ601 described in Davison et al Nucleic Acids Research 18 (No. 14):4285-5286, 1991.

VJS6

In this construct, containing the E6 protein from Human Papilloma Virus (HPV) a pE/L promoter (the "synthetic superpromoter") is placed just upstream the p7.5 promoter controlling the β-Gal expression and oriented in the same direction.

Control Vaccine

Control vaccinia (crude 19, NP-VV) does not expressing β-Galactosidase.

Primary in vivo Responses and Secondary in vitro Responses

Female BALB/c mice, 8-12 weeks old, were obtained from the Animal Production Colonies, Frederick Cancer Research Facility, NIH, Frederick, MD. Primary lymphocyte populations were generated by injecting BALB/c mice i.v. or subcutaneously with 10^6-10^7 plaque forming units (pfu) of recombinant virus (2 doses in some cases). To assay for primary *in vivo* responses spleens were harvested on day six, dispersed into a single cell suspension and tested for their ability to lyse β-gal expressing and control targets in a six hour ^{51}Cr release assay. Secondary *in vitro* effector populations were generated by harvesting the spleens of mice 21 days after immunization with recombinant virus and culturing single cell suspensions of splencytes in T-75 flasks (Nunc, Denmark) at a density of 5.0 x 10^6 splenocytes/ml. with 1 μg/ml of antigenic peptide in a total volume of 30 ml of culture medium consisting of RPMI 1640 with 10% fetal calf serum (both from Biofluids) that contained 0.1 mM non-essential

amino acids, 1.0mM sodium pyruvate (both from Biofluids) and 5 x 10^{-5} M 2-mercaptoethanol (GIBCO/BRL, Rockville, MD) in the absence of IL-2. Six days later splenocytes were harvested and washed in culture medium before testing in a ^{51}Cr release assay.

^{51}Cr release assay

Six-hour ^{51}Cr release assays were performed as previously described (Restifo, N. et al. J. Exp. Med. 177:265, 1993). Briefly, 1 x 10^6 target cells were incubated with 200mCi $Na^{51}CrO_4$ (^{51}Cr) for ninety minutes. Peptide pulsed targets were incubated with 1 μg/ml (which is roughly 1μM) of antigenic peptide (for β-gal = TPHPARIGL peptide) during labeling as previously described (Restifo, N. et al. J. Immunol 147:1453, 1991). Target cells were then mixed with effector cells for six hours at the effector to target (E:T) ratios indicated. The amount of ^{51}Cr released was determined by γ-counting and the present specific lysis was calculated from triplicate samples as follows: [(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)] x 100.

In vivo protection and treatment studies

For *in vivo* protection studies, mice were immunized with recombinant virus 21 days before a subcutaneous challenge with 10^4 tumor cells or an intravenous challenge with 5 x 10^5 tumor cells, as previously described (31). After tumor challenge all mice were randomized. Mice receiving subcutaneous tumor were measured twice a week. When tumors developed, they all grew progressively and were lethal. Mice were euthanized, however, when they were moribund. All mice that appear as long term survivors had no palpable tumor. Mice receiving intravenously administered tumor were euthanized on day 12 and randomized before counting lung metastases in a blinded fashion as previously described (2).

For *in vivo* treatment studies, un-irradiated BALB/c

mice were challenged with either 10^5 or 5 x 10^5 tumor cells intravenously in order to establish pulmonary metastases. Mice were subsequently vaccinated with 10^7 PFU of the designated recombinant virus intravenously or subcutaneously on days three or six. Mice receiving intravenously administered tumor were euthanized on day 12 and randomized before counting lung metastases in a blinded fashion.

The following targets were used in each assay:

CT26 = murine H-2^d adenocarcinoma

CT26 gal = murine H-2^d adenocarcinoma expressing β-Gal. CT26 gal also expresses higher levels of class I MHC molecules.

E22 = murine H-2^d thymoma expressing β-Gal.

CT26 + peptide = CT26 pulsed with TPHPARIGL (876-884) L^d -restricted β-gal peptide

CT26-vac = CT26 infected with crude 19 vaccinia

Lytic Units 30% were calculated for the anti-vaccinia cytotoxic response. Lytic units 30% (L.U. 30) indicate the number of effector cells necessary to give a 30% lysis of 10,000 target cells.

Example 5

Cytokine Secretion After Infection Of BSC-1 Cells With Various Recombinant Vaccinia Virus Constructs

Duplicate wells of 10^5 BSC1 in 1 ml of 2.5% fetal calf serum (FCS) medium RPMI 1640 with antibiotics (24 well plates) were infected with VJS6, rVV-IL-2, rVV-GM-CSF, rVV-IFNγ or rVV-TNFα. At the end of incubation at 27°C for 24 hours, supernatants were removed, centrifuged, and the concentration of cytokine determined as shown in Table 1. Values are expressed in pg/ml.

The concentration of each cytokine was determined using commercially available detection kits for GM-CSF, IFNγ; IL-2 and TNFα.

The results show that high concentrations of each cytokine were detected. The highest concentration of cytokine produced was IL-2 from BSC-1 cells infected with rVV-IL2 (Table 1).

Table 1

DETECTION OF CYTOKINES SECRETED AFTER INFECTION OF BSC1 CELLS WITH DIFFERENT rVV

	MOI 12h				MOI 36h			
rVV	1:1	0.1:1	0.01:1	TITER[a]	1:1	0.1:1	0.01:1	TITER[a]
GM-CSF detection kit								
VJS6	<15.6	<15.6	<15.6	-	<15.6	<15.6	<15.6	-
IL-2	<15.6	<15.6	22	-	<15.6	<15.6	<15.6	-
GMCSF	>250	>250	>250	$5.1X10^5$	>250	>250	>250	$4.2x10^6$
IFNγ	<15.6	<15.6	<15.6	-	<15.6	<15.6	<15.6	-
TNFα	<15.6	<15.6	<15.6	-		<15.6	<15.6	-
IFNγ detection kit								
VJS6	<47	<47	<47	-	<47	<47	<47	-
IL-2	<47	<47	<47	-	<47	<47	<47	-
GMCSF	<47	<47	<47	-	<47	<47	<47	-
IFNγ	>12000	9000	1000	56000	>12000	>12000	>12000	>67000
TNFα	<47	<47	<47	-	<47	<47	<47	-
IL-2 detection kit								
VJS6	<34	<34	<34	-	<34	<34	<34	-
IL-2	>850	>850	>850	$2x10^6$	>850	>850	>850	$3.9x10^6$
GMCSF	<34	<34	50	-	54	63	110	-
IFNγ	<34	121	135	-	94	97	132	-
TNFα	<34	<34	<34	-	<34	<34	<34	-
TNFα detection kit								
VJS6	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
IL-2	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
GMCSF	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
IFNγ	<30.2	<30.2	<30.2	-	<30.2	<30.2	<30.2	-
TNFα	2050	310	295	2050	>2450	>2450	>2450	135000

a. Supernatants from 1:1 MOI (multiplicity of infectivity) infected cells, that were expected to exceed the highest point of the standard curve, were serially diluted (1:10 dilution) to calculate the precise cytokine concentration.

b. Only one of the two wells was positive.

Example 6

β-Galactosidase Production After BSC1 Cell Infection With Various Vaccinia Virus Constructs

One X10^5 BSC1 cells were infected with 1:1 MOI of either rVV-VJS6, rVV-IL2, rVV-GM-CSF- rVV-TNFα or rVV-IFNγ. At this MOI the cytopathic effect was less pronounced at time end of incubation, and presumably no leakage of the enzyme had occurred. Pelleted cells were subject to three freeze-thaw rounds to release the cytoplasm content, cellular debris was removed and the supernatant used for detection of galactosidase activity on the substrate O-nitrophenyl-β-D-galactopyranoside (ONPG) using a Promega Kit No. E2000 (Promega, Madison, Wisconsin). One unit of galactosidase hydrolyze 1μM ONPG per minute at pH 7.5 at 37°C. Duplicate wells were run on each sample. The results from two different experiments are shown in Figure 1. The control vaccinia, NP-VV, did not express β-gal. VJS6 samples were serially diluted to fit within the depicted range. The results show a 1 log difference in the β-Gal enzymatic activity of VJS6 compared to the other recombinant vaccinia virus. The results as shown in Figure 1 parallel with the kinetics of blue staining with X-gal in plaque assays **(data not shown)**.

Example 7

Primary Response Of Mice At Day 6 After Injection I.V. With Various Vaccinia Virus Constructs

5 BALB/c mice were injected intravenously (I.V.) with 5x10^6 plaque forming units (pfu) of the following recombinant vaccinia virus: VJS6, IL2-rVV, GM-CSF-rVV, TNFα-rVV OR IFNγ-rVV. At day 6, the spleens were removed and tested against the following target cells as described in Example 4: CT26, CT26 peptide, CT26 gal, E22, and CT26 vac.

Figure 2 shows the peak primary CTL response. IL-2 and GM-CSF produced by rVV during infection notably

enhanced the primary CTL response against vaccinia determinants. The spleen cells also gave a weak primary response toward β-gal.

Example 8

Secondary Cultures From Mice Infected I.V. With Various Recombinant Vaccinia Virus Constructs

Mice were infected with various recombinant vaccinia virus constructs as described in Example 4. Splenocytes were harvested at day 6 or day 14 after inoculation with the rVV. Secondary cultures were generated by 6 days incubation of $6x10^6$ splenocytes/ml in complete medium containing 1 μg/ml TPHPARIGC peptide as indicated in Example 4. The results showed that CTL from VJS6, IL2-rVV and GM-CSF-rVV vaccinated mice could not be restimulated with an β-gal peptide 6 days after viral injection (Figure 3). The TNF-rVV response of CTLs was characterized by the induction of a long-lasting non-specific cytotoxicity (Figures 3 and 4). The GM-CSF production had a negative effect on the anti-β-gal immune response of CTLs.

Example 9

Primary Response Of Mice At Day 6 After Injection S.C. With Various Recombinant Vaccinia Virus Constructs

5 BALB/c mice were injected subcutaneously (S.C.) with $5x10^6$ pfu of the following recombinant vaccinia virus: VJS6, IL2-rVV, GM-CSF-rVV, TNFα-rVV or IFNγ-rVV. At day 6, the spleens were removed and tested against the following target cell as described in Example 4: CT26, CT26 peptide, CT26 gal, E22 and CT26 vac.

Figure 5 shows in all cases, each cytokine helped to increase the response to vaccinia in comparison to VJS6 using the CT26 target, with the IL-2-rVV treatment being the highest. No lysis was obtained with the other target cells. This indicates that the kinetics of the immune response induced by S.C. inoculation may be different or delayed in comparison to I.V. inoculation. However,

higher doses and/or different time intervals is expected to increase the magnitude of the primary response against vaccinia using the subcutaneous route of inoculation.

The lytic units (L.U.) 30% was determined by the number of effector cells necessary to give 30% lysis of 10,000 target cells using the CT26-vac cells as targets. Figure 6 shows the results of this titration. The L.U./spleen ($x10^3$) of each rVV is as follows: Gm-CSF= 124.3; VJS6 - 14.9; IFN-γ - 5.06; TNFα = 2.88 and IL-2 = 96.2.

Example 10

Secondary Cultures From Mice Infected S.C. With Various Recombinant Vaccinia Virus Constructs

Mice were infected with various recombinant vaccinia virus constructs as described in Example 4. Splenocytes were harvested at day 14 after inoculation with rVV. Secondary cultures were generated by 6 days incubation of $6x10^5$ splenocytes/ml in complete medium containing 1 μg/ml TPHPARIGC peptide as indicated in Example 4. The results as depicted in Figure 7 show a high level of non-specific response to each target cell regardless of the effector cell.

Example 11

Treatment Of Established Pulmonary Metastases With Recombinant Vaccinia Viruses Secreting Different Cytokines

The three-day pulmonary metastases mouse model was used to evaluate the efficacy of treatment using rVV. Nontreated mice normally succumb to the metastases in 11-14 days using this model system.

Mice were injected I.V. on day 0 with $5x10^5$ tumor cells (CT26 or CT26 β gal). Three days later they received an I.V. injection of $5x10^5$ PFU of VJS6, IL-2-rVV, GM-CSF-rVV, IFNα-rVV, TNFγ-rVV or Hanks' Balanced Salt Solution (HBSS) (control mice). The mice were randomized, and the lungs were harvested after 12 days. The number of

metastases was determined by counting in a blinded fashion.

Table 2 shows that moderate doses of IL2 produced by the rVV was effective in treatment of 3 day old metastases induced by CT26 β gal.

Table 2

DAY 3 TREATMENT OF ESTABLISHED PULMONARY METASTASES WITH RECOMBINANT VACCINIA VIRUSES SECRETING DIFFERENT CYTOKINES

	CT26		CT26 βgal	
rVV treatment	average # metastases	metastases/ mouse	average # metastases	metastases/ mouse
none*	>500	>500 x 5	>500	>500 x 5
VJS6	>500	>500 x 5	186	162, 116, 115, 362, 175
IL-2 rVV	452.4	500 x 4, 262	11.4	13, 2, 24, 12, 7
GM-CSF rVV	470.2	500 x 4, 351	373.6	500 x 2, 405, 283, 179
IFNγ rVV	>500	>500 x 5	232.8	280, 97, 190, 260, 337
TNFα rVV	>500	>500 x 5	361.2	>500 x 2, 389, 258, 159

* control mice were injected with HBSS alone

Example 12

Treatment Of Established Pulmonary Metastases With Recombinant Vaccinia Virus Secreting Different Cytokines Plus Exogenous IL2

Mice were injected with tumor cells and rVV as indicated in Example 4. Treatment with exogenous rIL-2 (15,000 cetus units, twice a day, I.P.) was started 6 h after rVV injection and protracted for 5 days. Lungs were harvested after 12 days.

Moderate doses of IL2 (produced by rVV or exogenously administered together with rVV) were effective in treatment of 3 day old metastases induced by CT26 β- gal. (Table 3)

Table 3

DAY 3 TREATMENT OF ESTABLISHED PULMONARY METASTASES WITH RECOMBINANT VACCINIA VIRUSES PRODUCING DIFFERENT CYTOKINES PLUS EXOGENOUS IL-2

	CT26		CT26 βgal	
rVV treatment	average # metastases	metastases/ mouse	average # metastases	metastases/ mouse
none[a]	>500	>500 x 5	>500[b]	>500 x 5
VJS6	>500	>500 x 5	405.2	>500 x 3, 267, 259
IL-2 rVV	437.4	500 x 3, 267, 420	127.4[c]	184, 7, 126, 91, 229
rIL-2	>500	>500 x 5	>500	>500 x 5
rIL-2 + VJS6	406.4	>500 x 3, 298, 234	20.8[c]	43, 6, 0, 52, 3
GM-CSF rVV	413.2	>500 x 3, 163, 403	500	500 x 5
IFNγ rVV	447.6	>500 x 4, 238	412.2	500 x 4, 66

[a] control mice were injected with HBSS alone
[b] all mice in this group died between days 11 and 12 (before lung harvest)
[c] p value between the data in the frame is 0.07

Example 13

Active Immunotherapy Using Exogenous IL-2 Together With rVV Expressing A Tumor Associated Antigen

A recombinant vaccinia virus expressing a Tumor Associated Antigen (TAA) was constructed as described in Example 1.

Mice were injected with 5 x 10^5 of CT-26 expressing β-gal via an I.V. route of administration. Three days later the mice were injected with rVV-TAA-β-gal, alone or in combination with exogenous IL2. [High dose (HD) exogenous IL-2 (100,000 I.U., I.P., BID x 3d) or low dose (IL) IL-2 (15,000 I.U., I.P., BID x 3d)] was administered as indicated and then randomized.

The number of mice surviving and survival time in days was monitored. Those mice receiving rVV-TAA plus exogenous IL2 survived. (Figure 8)

Example 14

Figure 9 shows the survival of mice with non-β-gal expressing tumor established after immunotherapy with different recombinant vaccinia vectors. Mice were injected with 5 x 10^5 CT 26 tumor cells (non-β-gal) via I.V. route of administration. On day three they were injected with 10^3 pfu of the following rVV which also contained the β-gal gene: IL-2rVV, TNFα-rVV, VJS6, GM-CSFrVV. The number of mice surviving and survival time in days was monitored. No difference in survival was observed in any of the treatment groups.

Figure 10 shows survival in mice with an established β-gal expressing tumor, CT26.25 after immunotherapy with different recombinant vaccinia vectors. Mice were injected with 5 x 10^5 CT26.25 (β-gal expressing) via I.V. route of administration. On day three they were injected with 10^3 pfu of the rVV described above. The results show that a clear survival advantage was conferred on those mice treated with the IL-2rVV (also containing the β-gal gene) compared to treatment with any other rVV.

Example 15

Active Treatment Of Established
Pulmonary Metastases Using
B7 - rVV Construct

All of the following recombinant Vaccinia vectors were constructed by placing both the cytokine gene and the TAA gene in the TK region of the vaccinia vector genome. Promotors varied based on the plasmid used for recombination. The majority of the constructs used the P7.5K promotor.

The three-day pulmonary metastases model was used as described in Example 4. Five x 10^5 tumor cells were injected into mice. Three days later 10^6-10^7 pfu of each vector was injected I.V. Lungs were harvested on day 12.

The results as depicted in Figure 11 and 12 showed that the rVV vectors encoding ß-gal and the

immunostimulatory molecule, B7.1, IL-2 or ICAM-1 profoundly inhibited pulmonary metastases in an antigen specific manner.

The primary response of the mice on Day 9 after injection with tumor cells was determined. The results are depicted in Table 4. The percent lysis of ^{51}Cr labeled target tumor cells is recorded. A significant primary immune response was seen with multiple recombinant vaccinia vectors, most notably B7.1rVV, which correlates with the in vivo treatment response.

Table 4
Primary Response of Mice 6 Days After Injection I.V. With Various Vaccinia Virus Constructs

		Tumor cells			
rVV (pfu) Construct	Effector Target	CT26	CT26/P	C25	CT26/V69
B7.1	100:1	-3.86*	82.99	81.45	21.69
	33:1	-4.86	91.86	69.01	14.49
	11:1	-6.10	61.64	71.75	7.33
	37:1	-8.61	53.99	54.22	2.19
	1.2:1	-9.53	39.93	40.87	3.25
	04:1	-16.57	27.37	26.74	-0.15
B7.1 10^6	100:1	2.75	68.18	5270	70.75
	33:1	-2.05	84.93	66.76	82.89
	11:1	-2.85	41.27	73.12	56.05
	37:1	-3.76	41.93	350.06	42.19
	12:1	-8.05	26.24	31.70	44.97
	0.4:1	-8.52	20.50	35.49	37.13
NA 10^7**	100:1	4.04	79.02	69.65	14.06
	33:1	1.70	72.66	82.36	10.65
	37:1	-1.01	51.75	63.41	7.53
	11:1	-2.75	35.65	42.33	0.37
	12:1	-4.88	34.98	28.49	-11.67
	0.4:1	-6.57	6.14	20.02	-13.66
NA 10^6	100:1	27.40	88.40	121.19	31.48
	33:1	13.47	74.16	112.42	1.41
	11:1	0.53	41.16	97.33	-4.07
	3.7:1	-0.35	8.95	86.27	1.58
	1.2:1	-0.43	-2.05	69.52	371
	04:1	-4.06	-2.59	40.13	-4.37

MVA 10^8	100:1	12.76	27.43	65.08	12.10
	33:1	8.52	17.62	61.83	8.37
	11:1	3.45	2.95	49.38	4.34
	37:1	0.06	5.76	28.12	3.13
	1.2:1	-1.30	2.43	28.05	-3.60
	0.4:1	-4.26	-0.61	16.78	-7.65
MVA 10^7	100:1	17.54	71.07	46.31	0.14
	33:1	13.81	70.97	37.10	1.10
	11:1	6.36	44.59	35.00	-0.30
	3.7:1	-2.29	17.29	28.58	-6.94
	1.2:1	-3.54	2.82	27.91	-0.76
	0.4:1	-17.53	-6.63	19.66	-9.87
Kb 10^7	100:1	24.83	20.58	33.37	34.47
	33:1	20.74	15.35	19.42	34.75
	11:1	12.61	-10.25	9.73	27.77
	37:1	9.82	-11.37	8.51	17.51
	1.2:1	-5.98	-18.23	8.58	17.01
	0.4:1	-10.88	-29.04	5.55	9.10
Kb 10^6	100:1	11.60	28.14	42.96	-0.32
	33:1	-2.37	16.79	21.60	-1.60
	11:1	-7.83	16.12	-1.67	-7.08
	37:1	-12.70	8.51	-3.28	-11.12
	1.2:1	-18.17	6.15	-11.68	-9.36
	0.4:1	-24.72	-4.83	-17.02	-14.28
Ld 10^7	100:1	-9.69	86.06	92.89	-5.1
	33:1	-32.65	33.05	53.22	-8.54
	11:1	-39.51	35.26	46.63	-19.29
	37:1	-42.44	15.50	24.28	-17.91
	1.2:1	-47.50	2.17	12.71	-17.45
	0.4:1	-51.77	-1.65	9.44	-30.42
Ld 10^6	100:1	28.10	56.29	30.10	16.62
	33:1	16.09	41.69	26.49	-5.75
	11:1	-1.22	24.49	24.50	-17.06
	37:1	-3.46	24.04	18.81	-21.05
	1.2:1	-9.65	8.65	13.16	-20.82
	0.4:1	-17.10	2.42	8.34	-27.53
VJS6 10^7	100:1	29.38	33.59	73.98	22.03
	33:1	24.69	31.48	72.14	24.43
	11:1	20.17	27.87	61.82	13.66
	37:1	16.83	23.47	57.46	7.24

	1.2:1	8.44	17.93	51.60	4.11
	0.4:1	4.01	19.12	33.13	-5.46
VJS6 10^6	100:1	1.53	63.88	63.8	45.57
	33:1	-23.69	35.15	36.13	39.89
	11:1	-27.32	29.65	27.61	29.29
	37:1	-41.54	21.54	21.57	24.26
	1.2:1	-45.02	18.21	16.21	21.06
	0.4:1	-52.85	14.18	8.78	18.50
WB_2m 10^7	100:1	3.50	11.12	23.52	4.69
	33:1	0.32	11.95	10.52	3.46
	11:1	-10.09	5.10	7.51	-2.53
	37:1	-21.06	0.22	1.10	-2.51
	1.2:1	-28.28	-3.99	-3.04	-4.26
	0.4:1	-30.42	-7.84	-11.03	-7.36
WB_2m 10^6	100:1	25.09	16.18	22.86	1.54
	33:1	25.61	6.04	19.48	-2.55
	11:1	22.77	-1.80	14.43	-11.26
	37:1	9.18	-8.25	11.40	-19.36
	1.2:1	3.90	-19.60	10.69	-24.29
	0.4:1	-13.93	-28.67	-1.28	-29.35
HBSS	100:1	6.00	17.33	6.73	-4.50
	33:1	0.30	14.46	6.58	-9.50
	11:1	-10.32	9.65	-9.60	-5.68
	37:1	-14.34	1.66	-17.73	-13.69
	1.2:1	-23.70	-9.03	-30.78	-17.72
	0.4:1	-29.28	-12.56	-38.08	-17.75

* % lysis of ^{51}Cr labeled tumor cells
** NA = neuraminidase recombinant viral vector (rVV), MVA = Ankara non-replicating vaccinia virus rVV, Kb = murine H-$2K^b$ MHC Class I molecule rVV, Ld = murine H-$2L^d$ MHC Class I molecule rVV, WB_2m = murine Beta 2-microglobulin rVV, HBSS = Hank's Balanced Salt Solution

The secondary response of the mice is depicted in Table 5. The results indicate a secondary immune response seen by both VJS6 and B7.1 against the relevant target.

Table 5
Secondary Response of Mice 6 days After
I.V. Injection with Various Vaccinia Virus Constructs

		Tumor cells			
rVV (pfu) Construct	Effector Target	CT26	CT26/P	C25	CT26/V69
HBSS	100:1	30.26*	45.93	37.05	29.95
	33:1	15.83	13.38	10.05	18.24
	11:1	2.59	5.88	4.37	11.39
	37:1	0.83	5.64	-1.68	-1.00
	1.2:1	0.01	2.14	-2.71	4.12
	0.4:1	1.66	6.71	8.61	-0.11
VJS6 10^7	100:1	19.40	72.31	78.94	28.42
	33:1	21.52	83.69	63.82	18.04
	11:1	10.84	62.35	61.87	16.80
	37:1	7.91	44.31	52.99	4.75
	1.2:1	10.24	21.03	18.30	5.72
	0.1:1	4.59	14.14	11.92	10.07
VJS6 10^6	100:1	18.55	48.89	38.21	15.27
	33:1	11.33	41.52	34.52	8.25
	11:1	8.06	26.63	33.00	1.17
	37:1	2.57	16.54	29.26	-2.23
	1.2:1	7.86	14.96	13.57	0.56
	0.4:1	2.05	3.65	9.47	-8.17
Ld 10^7**	100:1	0.11	30.56	25.05	-4.91
	33:1	-2.78	25.03	26.24	-0.48
	11:1	2.49	16.01	11.97	-3.99
	37:1	-1.02	7.17	7.28	3.50
	1.2:1	0.52	9.57	4.36	4.06
	0.4:1	3.10	6.23	2.35	3.29
Ld 10^6	100:1	18.65	25.02	14.24	11.70
	33:1	0.63	11.23	7.51	11.36
	11:1	-0.35	7.47	10.24	2.76
	37:1	-0.47	1.38	-0.00	3.32
	1.2:1	-3.19	6.35	5.89	6.16
	0.4:1	4.08	5.70	2.86	4.04
B7.1 10^7	100:1	-10.62	83.64	69.56	-3.17
	33:1	0.20	63.61	59.63	0.23
	11:1	-6.10	40.58	61.77	3.02
	37:1	-1.91	22.29	22.86	-2.5
	1.2:1	-1.38	8.0	10.25	0.22

	0.4:1	4.73	8.39	9.49	2.22
B7.1 10^6	100:1	30.59	59.22	61.29	32.13
	33:1	31.24	74.4	53.99	23.29
	11:1	13.92	41.68	49.63	22.94
	37:1	10.66	31.02	34.86	5.63
	1.2:1	4.87	33.61	6.45	3.73
	0.4:1	8.78	7.66	3.98	7.16
MVA	100:1	28.16	18.13	25.09	30.30
	33:1	33.76	28.62	27.99	20.10
	11:1	28.69	12.63	9.75	9.13
	37:1	6.55	-0.43	12.79	2.78
	1.2:1	8.08	4.47	1.63	-0.45
	0.4:1	5.68	-1.30	1.21	4.60
MVA 10^7	100:1	22.77	32.85	28.05	27.07
	33:1	10.46	28.94	30.49	8.80
	11:1	5.48	24.20	22.32	-3.73
	37:1	0.49	11.9	12.82	4.36
	1.2:1	-0.95	5.29	13.32	6.36
	0.4:1	4.75	-0.73	14.69	9.59

* % lysis of ^{51}Cr labeled tumor cells
** NA = neuraminidase recombinant viral vecot (rVV), MVA = Ankara non-replicating vaccinia virus rVV, Kb = murine H-$2K^6$ MHC Class I molecule rVV, Ld = murine H-$2L^4$ MHC Class I molecule rVV, WB_2m = murine Beta 2-microglobulin rVV, HBSS = Hank's Balanced Salt Solution

Example 16

Clinical Protocol For Phase I/II Trials In Patients With Metastatic Melanoma and Metastatic Breast Cancer of Immunization with a Recombinant Vaccinia Virus Expressing the MAGE-1 Peptide and B7.1

In this protocol patients with advanced melanoma and advanced breast cancer are immunized against MAGE-1, an immunodominant peptide from a cancer antigen in combination with costimulatory/accessory molecule B7-1.

Patient Eligibility

Among other criteria, patients must have evidence of measurable or evaluable metastatic melanoma or breast cancer that has failed standard effective therapy. Patients must have tumors that express the MAGE-1 antigen

as evidenced by PCR or Northern Blot analysis of tumor cell RNA.

Treatment

Melanoma and breast cancer patients are divided into two categories each, those who have previously been vaccinated against smallpox and those who have not. Three patients in each category receive intradermal immunization with 10^8 plaque forming units/ml every four weeks for a total of three doses. Patients are evaluated for toxicity. When three patients in each category have been followed for at least two weeks after the first immunization without achieving grade 3 or 4 toxicity not easily reversible by standard measures then the dose in that plaque category will be escalated to 10^9 plaque forming units/ml intradermally every four weeks for a total of three doses. Fifteen patients in each category will be treated at this dose and carefully evaluation of toxicity, immunologic effects and therapeutic efficacy will be evaluated.

If any patient receiving the 10^8 pfu/ml dose achieves grade 3 or 4 toxicity not easily reversible by standard measures, then an additional three patients will be treated at that dose. If a second patient develops grade 3 or 4 toxicity, not easily managed by standard procedures, then the dose will not be escalated in that category.

Vaccine preparation. The recombinant vaccinia virus used in this protocol is the Wyeth vaccinia virus derived from the New York City Board of Health strain. This is an attenuated virus with an extensive history of previous use in humans. The recombinant virus has the gene coding for the MAGE-1 nine amino acid minimal determinant plus the B7.1 sequence inserted into the viral thymidine kinase gene.

The recombinant virus is produced in a Food and Drug Administration approved facility for the manufacture of GMP grade clinical material.

Vaccination procedure. The recombinant vaccinia virus will be supplied as a sterile, frozen suspension and will be approximately diluted to either 10^8 or 10^9 plaque forming units/ml with phosphate buffered saline.

Vaccination will be performed in the deltoid muscle area. The method of vaccination is summarized as follows:

a) Open the bifurcated needle by catching the butt-end of the needle and gently pulling the point end free.

b) Dip the bifurcated point of the needle into vaccine. The needle will pick up a drop of vaccine in the space between the two points.

c) Using the same bifurcated needle, use the multiple pressure technique by pressing the needle through the vaccine drop on the subject's skin. Fifteen needle pressures will be administered.

d) Alternative upper extremities will be used for subsequent vaccinations.

Patients will be observed for 24 hours after immunization. Body temperature will be measured at 12 hours and at 24 hours and any adverse reactions noted.

Post vaccination evaluation

On day 14 after vaccination, patients will be seen and the following tests obtained:

a) Complete history and physical examination, including any symptoms and progress of reaction from the vaccination.

b) Photograph of the vaccination site.

c) Complete blood count with differential count.

d) Platelet counts.

e) Acute care, hepatic, mineral and thyroid blood chemistry panel.

f) Serum and lymphocytes stored as performed

pretreatment.

Patients will be evaluated at 14 days following the second vaccination and similar procedures will be performed prior to and following the third and final vaccination on day 56.

Patients will have a complete restaging of all sites of disease with appropriate physical examination and x-rays and nuclear medicine studies at the time of one and two months following the final vaccination.

Immunologic studies

Immunologic assessment will be made of the patient's response to vaccinia antigens as well as to the MAGE-1 antigen.

a) All serum samples will be tested for anti-vaccinia antibody by ELISA.

b) All cryopreserved lymphocytes will be tested for response to MAGE-1 antigen using limiting dilution analysis of precursor CTL frequency using the method of Coulie, P et al International Journal of Cancer 50:289-297, 1992.

c) Patients with easily accessible disease may have biopsy under local anesthesia of accessible tumor to study the histopathologic nature of the tumor as well as the isolation of tumor infiltrating lymphocytes for in vitro growth. Tumor infiltrating lymphocytes will be tested for specific reactivity and specific cytokine release against MAGE associated antigens.

Assessment of response

A complete response is defined as the disappearance of all clinical evidence of disease that lasts at least four weeks.

A partial response is a 50% or greater decrease in the sum of the products of the perpendicular diameter of all measurable lesions for at least four weeks with no appearance of new lesions or increase in any lesions.

Minor responses are defined as 25-49% decrease in the sum of the products of the perpendicular diameters of all measurable lesions with no appearance of new lesions and no increase in any lesions.

Any patient with less than a partial response will be considered a non-responder.

The appearance of new lesions or greater than 25% increase in the product of perpendicular diameters of prior lesions following a partial or complete response will be considered as a relapse.

Similar protocols will be followed for the evaluation of other recombinant virus vaccines against cancer. Vaccines to be tested include but are not limited to Recombinant Vaccinia virus encoding MART-1 and IL-2, Recombinant Vaccinia virus encoding MART-1 and B7.1, Recombinant Vaccinia virus encoding GP100 and IL-2, Recombinant vaccinia virus encoding GP100 and B7.1, Recombinant fowlpox virus encoding MART-1 and IL2, recombinant fowlpox virus encoding MART and B7.1 and the like with the appropriate modifications depending on the antigen and virus used.

Example 17

Use of Lymphocytes Sensitized To Immunogenic Peptides Derived From Melanoma Antigens For Therapeutically Treating Mammals Afflicted With Melanoma

T-lymphocytes presensitized to the melanoma antigen may be effective in therapeutically treating mammals afflicted with melanoma. T-lymphocytes are isolated from peripheral blood or melanoma tumor suspensions and cultured *in vitro* (Kawakami, Y. et al. (1988) *J. Exp. Med.* 168:2183-2191). The T-lymphocytes are exposed to cells infected with the recombinant virus expressing a melanoma associated antigen and IL2 and/or B7.1 for a period of about to 1-16 hours at a concentration of 1 to 10mg/ml. T-lymphocytes exposed to the antigen will be administered

to the mammal, preferably a human at about 10^9-10^{11} lymphocytes per mammal. The lymphocytes may be administered either intravenously, intraperitoneally or intralesionally. This treatment may be administered concurrently with other therapeutic treatments such as cytokines, radiotherapy, surgical excision of melanoma lesions and chemotherapeutic drugs, adoptive T lymphocyte therapy.

Example 18

Active Immunotherapy with Recombinant Vaccinia Virus Co-expressing B7-1, B7-2 or Both and a Model Tumor Antigen Mediates Regression of Established Pulmonary Metastases

Materials and Methods

Animals

Female BALB/c (H-2^d) mice were obtained from Frederick Cancer Research Center (Frederick, MD). All mice were used at 6 - 8 weeks of age.

Preparation of Cell Lines

CT26 is an N-nitroso-N-methylurethrane induced BALB/c (H-2^d) undifferentiated colon carcinoma as supplied by D. Pardoll (Baltimore, MD). CT26 was cloned to produce a wild type parental tumor line, CT26.WT. The gene for *lac Z* was stably transfected into CT26.WT as previously described herein and in Wang, M. et al J. Immunol. 154(9):4685-4692, 1995. Briefly, a plasmid donated by A.D. Miller containing the gene for β-galactosidase and a neomycin resistance marker were used to construct the LZSN amphotropic retrovirus which was used to transduce CT26.WT. Transductants were selected in G418 media, and subcloned by limiting dilution analysis at 0.3 cells/well. Subclones that expressed β-galactosidase were screened by X-gal staining and in ^{51}Cr release assays with anti-β-galactosidase effectors. The subclone CT26.CL25 was selected for use in all studies because of its stable expression of both β-galactosidase and the class I

molecule H-2 L^d. BS-C-1 or HeLa S^3 cells (ATCC, American Tissue Typing Collection, Rockville, MD CCL 16) were used to expand and titer all viruses. Cell lines were maintained in RPMI 1640, 10% heat inactivated FCS (Biofluids, Rockville, MD), 0.03% L-glutamine, 100 μg/ml streptomycin, 100 μg/ml penicillin and 50 μg/ml gentamicin sulfate (NIH Media Center). CT26.CL25 was maintained in media containing 400 or 800 μg/ml G418 (GIBCO, Grand Island, N.Y.).

Construction and Characterization of Recombinant Vaccinia Viruses

Construction and characterization of recombinant vaccinia viruses containing murine B7-1(v.MCB7-1), B7-2 (v. MCB7-2), B7-1 and B7-2 (v.B7-1/B7-2) and measles hemagglutinin (HA) (v.MCMHA) is as follows. Briefly, the B7-1 gene (a gift from Dr. R. Germain, NIH) and the measles HA gene (a gift from Dr. S. Rozenblatt, Tel Aviv University, Israel) were cloned into the transfer plasmid pRB12 such that they were under control of the VV synthetic early/late promoter. The gene of interest was inserted into the Hind III F region of VV. Methods used for the production and selection of rVV in which the VV thymidine kinase gene was utilized as an insertion site, were similar to those described previously by Earl, P.L. et al. In: Current Protocols in Molecular Biol. Ausubil et al (eds) Greene Publishing Assoc. and Wiley Interscience, 1991, 16.18.1-16.18.10 unless otherwise stated. The v.MCB7-1/β-gal and v.MCMHA/β-gal were constructed by homologous recombination of the plasmid pSC65Δ (a modification of pSC65 in which the E. coli *lac Z* gene is under transcriptional control of the early function of the VV p 7.5k promoter and flanked by VV derived TK DNA) with v.MCB7-1 and v.MCMHA respectively. The v. MCB7-2/β-gal was constructed with both the B7-2 gene and the E. coli *lac Z* gene under transcriptional

control of the early function of the VV p 7.5k promoter and flanked by VV derived TK DNA. The v. MCB7-1/B7-2/β-gal was constructed by homologous recombination of the plasmid pSC65Δ (modified as stated previously to create the v. MCB7-2/β-gal construct) with the v. MCB7-1.

Table 6. Construction of recombinant vaccinia viruses.

Recombinant Vaccinia Virus Code	VP37		Thymidine Kinase	
	Promoter	Gene	Promoter	Gene
v.MCB7-1/β-gal	S.E/L	B7-1	7.5e	β-gal
v.MCMHA/β-gal	S.E/L	MHA	7.5.e	β-gal
v.MCB7-1/NP	S.E/L	B7-1	7.5e	NP
v. MCB7-2/β-gal			7.5e	B7-2, β-gal
v. MCB7-1/B7-2/β-gal	S.E/L	B7-2	7.5e	B7-2, β-gal
v.MCMHA/B7-2/B-gal	S.E/L	MHA	7.5e	B7-2, β-gal

MHA= measle hemagglutinin gene, B7-1 = murine B7-1, B7-2 = murine B7-2, NP = influenza nucleoprotein, β-gal= E.coli β-galactosidase gene, S.E/L = vaccinia synthetic early late promoter, 7.5e = early only function of the vaccinia 7.5k promoter.

Recombinant viruses were simultaneously selected for their TK negative phenotype and ability to express β-galactosidase. Transfer plasmid pGS69 (Smith, G.L. et al Virology 160:336-345, 1987 containing the influenza nucleoprotein (NP) was recombined with v.MCB7-1 to yield v. MCB7-1/NP. This recombinant virus was selected on the basis of a TK negative phenotype. Plaques were then analyzed for expression of NP by immunostaining (Sutter, G. et al Vaccine 12:1032-1040, 1994) and subsequently plaque purified under agar overlay four times before sucrose cushion purified viral stocks were prepared (Earl, P.L. et al Current Protocols in Mol. Biol., ibid (Table

6.).

Preparation of rVV expressing the influenza A/PR/8/34 nucleoprotein (NP) was previously described (Smith, G.L. et al Virology 160:336-345, 1987). In the control construct, v. JS6, the E.coli *lac Z* gene was under the control of the early VV p 7.5k promoter from plasmid pSC65. Murine IL-2 cDNA was amplified by polymerase chain reaction from pBMGNeomIL-2 and ligated into the *Sma I-BamHI* site of a vaccinia expression vector, pMJ601, which contains the b-galactosidase gene under the control of VV early p 7.5k promoter. The other cytokines (GM-CSF, IFN-g, and TNF-a) were inserted into the wild type VV genome with similar procedure. The techniques used in preparation of the v. HLA.A2.1/β-gal (O'Neil, B.H. et al J. Immunol. 151:1410-1418, 1993) v. human and murine β_2-microglobulin/β-gal, (O'Neil, B.H. et al J. Immunol. 151:1410-1418, 1993) and all the murine MHC I (L^d, D^d, K^d, and K^b)/β-gal (Restifo, N.P. et al J. Exp. Med. 177:265-272, 1993), has been previously described.

Analysis of Recombinant Proteins and Quantitation of β-galactosidase Expression

BS-C-1 monolayers were infected with 10 infectious units of recombinant virus per cell. After 20 hours infected cells were harvested in solubilization buffer (0.06M Tris-HCl (pH 6.8), 3% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue). Proteins were resolved on a 10% polyacrylamide gel and subsequently transferred to a nitrocellulose membrane using a Bio-Rad Transblott Mini cell (Bio-Rad) at 250 mAmps for 1 hour. Membranes were initially incubated for 1 hour in a blocking solution (PBS-B) consisting of PBS containing 3% non-fat milk (w/v) and 0.2% Tween 20. A murine specific B7-1 hamster antibody (supplied by Dr. H. Reiser, Dana-Farber Cancer Inst, Boston) was diluted in PBS-B and incubated with filters for 3 hours at room temperature.

Filters were subsequently washed three times in PBS containing 0.2% Tween 20. Bound primary antibodies were detected by incubating filters, after washing with ^{125}I-protein A (Amersham) at a concentration of 0.1μCi/ml in PBS-B. Filters were washed 4 times dried and exposed to X-ray film (Kodak). Protein sizes were estimated using 14C molecular weight markers (Amersham) (Fig. 13).

Recombinant viruses were analyzed for β-galactosidase expression using an enzyme assay kit (Promega, WI). Briefly, monolayers of BS-C-1 cells were infected at a multiplicity of infection (MOI) of 10. Approximately 20 hours post infection cells were washed with PBS and harvested with lysis buffer. Quantitation of enzyme expression was determined by incubating cell extracts with β-galactosidase substrate and buffer solution as described in the manufactureres protocol.

In Vivo Experiments

In vivo protection studies utilized BALB/c mice which were immunized intravenously with either Hanks's balanced salt solution (HBSS) (Biofluids, Inc., Rockville, MD), v.MCB7-1/b-gal, or v.MCMHA/b-gal (10^7plaque forming units (PFU)). Twenty-one days following this initial immunization, the mice were intravenously challenged with 5 x 10^5 tumor cells of CT26.WT or CT26.C25. Twelve days following tumor challenge, mice were randomized, euthanized, and lung metastases were enumerated in a blinded fashion.

In vivo adoptive transfer studies involved intravenous immunization of BALB/c mice with either HBSS, v.MCB7-1/b-gal or v.MC MHA/b-gal . The rVV was administered at either 10^5 or 10^7 PFUs. After twenty-one days, these mice were euthanized, and splenectomized. Following splenectomy, the spleens were morselized, passed through a Nytex membrane, and suspended in HBSS. Lymphocytes were counted on a hemocytometer, and 2 x 10^7 lymphocytes were adoptively transferred to mice who had

been injected intravenously with 5 x 10^5 tumor cells of either CT26.WT or CT26.C25. Designated groups of mice received adjuvant treatment with six doses of IL-2 administered intra-peritoneal at a dose of 100,000 Cetus units two times per day for three days. Nine days after adoptive transfer of primed splenocytes, the mice were randomized, euthanized, and lung metastases counted in a blinded fashion.

In vivo active treatment studies involved non-irradiated BALB/c mice which were inoculated intravenously with 5 x 10^5 tumor cells of either CT26.WT or CT26.C25. All mice were randomized and subsequently vaccinated with HBSS, or 10^5 or 10^7 PFUs of the designated rVV three or six days later. Designated groups of mice received adjuvant treatment with six doses of IL-2 administered intra-peritoneal at 100,000 Cetus units two times per day for three days. Mice were randomized and euthanized on day twelve, and lung metastases were counted in a blinded fashion. Identically treated groups of mice bearing three day tumor burdens were also followed long term to assess the effect of vaccination on survival.

T cell Subset Depletions

Purified culture supernatants of anti-CD4 monoclonal antibody GKI.5 (TIB 207; American Tissue Culture Collection) and ascitic fluid of hybridoma 2.43 (anti-CD8) (TIB 210: American Tissue Culture Collection) were diluted in HBSS prior to use *in vivo*. For *in vivo* depletion BALB/c mice were given i.v. injections of GK1.5 at 100 mg/ml or of empirically determined levels of 2.43 monoclonal antibodies 48 hours prior to receiving tumor challenge, and again 6 days later. Using fluorescein isothiocyante-labeled anti-CD4 and phycoerythrin labeled anti-CD8 antibodies, FACS analysis was performed 1 day prior to immunization, and again at day 7 to verify depletion.

Whole Organ X-gal Staining

Lungs from tumor-bearing mice were removed and inflated with PBS (Biofluids) prior to X-gal staining. Lungs were fixed in a solution containing 2% formaldehyde (v/v), 0.2% gluteraldehyde (v/v) in PBS for 45 minutes and washed with PBS three times. After washing, the lungs were stained with X-gal solution for 12 hours at 37° C. X-gal solution for whole organs was prepared by combining the following: 0.02% (v/v) NP-40, 0.01% (w/v) sodium deoxycolate, 1 mg/ml X-gal, 5 mM potassium ferrocyanide, 2 mM MgCl2 in PBS. After staining in X-gal solution, the lungs were rinsed briefly with 3% (v/v) dimethyl sulfate, and then with PBS and stored at 4° C in 0.02% (w/v) sodium azide in PBS.

Statistical Analysis

Data from *in vivo* protection, adoptive, and active treatment studies was analyzed using a two-sided student's *t* test for unpaired samples. Errors bars represent the standard deviation within each group. Survival was analyzed with standard Kaplan-Meier survival curves (Kaplan, E.L. J. Am. Stat. Assoc 53:457-481, 1958). All p values presented are two-sided.

Example 19

Characterization of Murine B7-1 and E.coli *ß*-gal Genes in Cells Infected by Vaccinia Viruses

Following preparation of the rVVs, and prior to their use in *in vivo* experiments, the the *in vitro* expression of foreign protein in infected cells was evaluated by either immunohistochemical staining, western blot analysis, or both. Western blot analysis of murine B7-1 expression of virally infected cells illustrated the previously reported diffuse staining pattern typical of highly glycosylated proteins (Fig. 13) (Freeman, G.J. et al J. Exp. Med. 174:625-631, 1991). Molecular weight markers estimate VV expressed B7-1 to be approximately 55-65 k Da

in size. The authenticity of rVV expressed murine B7-1 was further verified by the binding of CTLA4-Ig to v. MCB7-1 infected cells. Expression of rVV murine B7-2 was verified by immunohistochemical staining of infected cells using a murine specific B7-2 antibody (PharMingen, CA). Expression of the TAA, β-galactosidase, was similar for v. MCB7-1/β-gal and the control recombinant v. MCMHA/β-gal, in contrast v. MCB7-1/NP showed no signs of enzyme expression (data not shown).

Example 20

Initial Screen: Ability of Immunization with Recombinant Vaccinia Virus to Mediate Regression of Established Tumor

It was determined whether combining a TAA with one or several of these immunomodulatory molecules would enhance the antigen specific immune response, as measured by the most stringent test, i.e., the ability to mediate regression of established disease. The virus, v. JS6, expressing the TAA alone, was used as control to measure either the positive or negative contribution of the immunomodulatory molecule in the vaccination vector.

The ability of a single inoculation of twelve double rVVs, which expressed the genes encoding both an immunomodulatory molecule and the β-gal model tumor antigen in the same virus, to effect regression of pulmonary metastases in tumor-bearing mice was assayed. The double rVVs were constructed to include the b-galactosidase gene within the thymidine kinase (TK) region of the VV genome for all vectors evaluated. The position of the immunomodulatory molecule for most vectors is also within the TK region of the VV genome under the same promoter as the TAA. However, in the case of the B7-1 vector, the gene for B7-1 is placed within the Hind III F region as described above and shown in Table 6.

The double rVVs studied were v.MCB7.1 (Hind III F

region of VV genome)/b-gal (TK region of VV genome), v. human b_2-microglobulin (TK)/b-gal(TK) {3154}, v. murine b_2-microglobulin (TK)/b-gal(TK) (O'Neil, B.H. et al J. Immunol. 154:1410-1418, 1993), v. murine MHC I L^d (TK)/b-gal (TK) (Restifo, N.P. J. Exp. Med. 177:265-272, 1993), v. MHC I K^d (TK)/b-gal (TK) (Restifo, N.P. J. Exp. Med. 177:265-272, 1993), v. MHC I D^d (TK)/b-gal (TK), v. MHC I (allogeneic) K^b (TK)/b-gal (TK) (Restifo, N.P. J. Exp. Med. 177:265-272, 1993), v. human leukocyte antigen (xenogeneic) HLA-A_2 (TK)/b-gal (TK) (O'Neil, B.H. et al J. Immunol. 154:1410-1418, 1993), v. murine interleukin - 2 (IL-2) (TK)/b-gal (TK), v. murine granulocyte-macrophage colony stimulating factor (GMCSF) (TK)/b-gal (TK), v. interferon-gamma (IFN-g) (TK)/b-gal (TK) (Bronte et al., in press), and v. tumor necrosis factor-alpha (TNF-a) (TK)/b-gal (TK). The v. JS6, expressing only b-gal in the TK region (described above), and a non-replicating VV, modified vaccinia Ankara (MVA) (Sutter, G. et al Proc. Nat'l Acad. Sci. U.S.A. 89:10847-10851, 1992), also only expressing b-gal, were used as controls.

Using the *in vivo* active treatment protocol outlined above, mice bearing three day pulmonary metastases were immunized with a single dose of the selected rVV. In each experiment, no therapeutic response was seen with any rVV against the parental tumor, CT26.WT, thus documenting the specificity of the response. Figure 14 displays the results of our initial screening experiment in which mice bearing three-day CT26.C25 tumors were vaccinated with rVV (10^7 PFUs).

When compared with the HBSS vaccinated control mice, only v. IL-2/b-gal and v.MCB7-1/b-gal mediated both a specific and significant reduction in the number of pulmonary metastases ($p < 0.006$ and $p< 0.002$, respectively). The ability of v. JS6, the single rVV expressing b-gal alone, to mediate significant tumor regression was variable from experiment to experiment.

(Data not shown). The inability of v. MVA/b-gal, a non-replicating VV which produces high levels of b-gal, to mediate any regression, may indicate that prolonged TAA expression (as would occur with a replicating VV) is required for a successful CTL response. Treatment with v. IL-2/b-gal and v. MCB7-1/b-gal resulted in a 10 and 5 fold further reduction in the number of metastases when compared to the best results obtained with v. JS6. These results suggest that vaccination with an immunodominant TAA alone is capable of priming a cytotoxic antitumor immune response which can then be further enhanced by the simultaneous expression of unique immunomodulatory molecules.

Although all double rVV contained the gene for the TAA, b-gal, only v. IL-2/b-gal and v. MCB7-1/b-gal were able to mediate significant reductions in the number of pulmonary metastases. Insufficient b-gal expression is unlikely to account for the therapeutic failure of the ineffective double rVVs. When tested, b-gal expression, at least between the individual cytokine producing rVV, did not significantly different (data not shown). The potential for the gene encoding for the immunomodulatory molecule within the double rVV to exert an immunosuppressive effect on the generation of a TAA cytotoxic response, or directly inhibit TAA protein expression was not evaluated, and cannot be excluded. The possibility that the failure of the GM-CSF, IFN-γ, or TNF-α producing rVVs is due to immunosuppresive effects of local cytokine production was not further explored.

Tumor regression following vaccination with v. MCB7-1/b-gal has not been previously reported. However, the therapeutic result obtained with this rVV supports the notion that costimulatory molecule expression is necessary for optimal T cell activation. Of note, a single vaccination with v. D^d/b-gal at a lower viral inoculate (10^6 PFUs) was also capable of mediating a

significant reduction in the number of pulmonary metastases ($p < 0.005$). This suggests the existence of a D^d epitope for the b-gal protein heretofore unknown.

Based on the findings of this screen, and other reports of anti-tumor effects with both B7-1 transfectants (Guinan, E.C. Blood 84:3261-3282, 1994) and v. B7-1 oncolysates (Hodge, J.W. et al Cancer Res. 54:5552-5555, 1994), a more extensively evaluate the *in vivo* effects of v. MCB7-1/b-gal on the cell-mediated antitumor immune response was conducted. Interestingly, the generation of CTL following B7-1 transfection is an IL-2 dependent phenomenon, since the addition of IL-2 blocking antibody during the induction phase blunts generation of a cytotoxic response (Harding, F.A. et al J. Exp. Med. 177:1791-1796, 1993).

Example 21

Protection Against Subsequent Tumor Challenge by Immunization with v. MCB7-1/β-gal

Although the identification of murine and human TAAs and TAA specific CTLs confirm that an immunocompetent host can generate a significant cytotoxic T cell response against autologous neoplasms; the immunogenicity of these TAA is generally weak allowing for the progressive growth of tumors. Protective immunity following vaccination with a rVV expressing either the TAA alone, or in combination with B7-1 was evaluated. The rVV strategy utilized in this current study has a distinct advantage over B7 transfections studies (in addition to the ease of administration), since the specificity of the immune response against a defined model TAA can be measured by comparing the response in the transduced tumor cell line CT26.C25, with the parental tumor, CT26.WT. Whereas the protective immunity demonstrated in the transfection studies is interpreted as a specific CTL response against an undefined TAA, the unique reactivity of the CTL in this model is easily evaluable.

Naive mice were initially vaccinated with either v. MCB7-1/b-gal, or a control virus, v. MCMHA/b-gal. The v. MCMHA/b-gal vector expressed an irrelevant protein, measles HA, which is a similarly sized and glycosylated molecule like B7-1, as well as the model TAA to allow for evaluation of the contribution of B7-1 to the response generated. Mice immunized with both v. MCB7-1/b-gal and v. MCMHA/b-gal were protected against tumor challenge with CT26.CL25, but not the wild type tumor, CT26.WT ($p < 0.0002$ and $p < 0.0014$, respectively) (Fig. 15.). These results confirm previous reports that vaccination with a rVV expressing a unique TAA alone can confer protection to subsequent challenge with a TAA-expressing tumor. (Kantor, J. et al J. Nat'l Cancer Inst. 84:1084-1091, 1992; Estin, C.D. et al Proc. Nat'l Acad. Sci. U.S.A. 85:1052-1056, 1988). The completeness of the protection by both viruses precluded a more thorough evaluation of the impact of the B7-1 gene in this situation. In this model system, b-gal protein expression by both v. MCMHA/b-gal and v. MCB7-1/b-gal was sufficient to exploit intracellular antigen processing mechanisms leading to protective immunity.

Example 22

Primary Adoptive Transfer of v. MCB7-1/β-gal Primed Splenocytes to Tumor-Bearing Syngeneic Mice

Tumorigenicity is reflective of the state of T cell activation. In the present invention it was hypothesized that vaccination a rVV coexpressing B7-1 and a model TAA, would result in the generation of a primary TAA CTL response which could be used in the adoptive therapy of tumor-bearing syngeneic host. The therapeutic advantage gained by immunizing against a known TAA to elicit CTLs reactive against both autologous or allogeneic tumors are numerous. Confirmation of the specificity of the CTL response was demonstrated in the initial observation that no reduction in the number of pulmonary metastasis was

noted after the adoptive transfer of any group of primed lymphocytes to syngeneic mice bearing CT26.WT tumors.

Mice bearing CT26.C25 tumors and inoculated with HBSS primed splenocytes showed no therapeutic benefit, however the addition of adjuvant IL-2 to similarly treated mice resulted in a significant reduction in the number of pulmonary metastases ($p < .0054$) (Fig. 16.). CT26.C25 tumor-bearing mice treated with v. MCB7-1/b-gal rVV splenocytes at two doses (10^5 and 10^7 PFUS) showed a significant reduction in tumor burden ($p < 0.007$ and $p < 0.007$, respectively). Again, adjuvant IL-2 treatment resulted in a 16.5 and 12.5 fold further reduction in the number of pulmonary metastases in this group. Significant tumor regression was also seen in mice receiving splenocytes primed with v. MCMHA/b-gal at 10^5 PFU, but not at 10^7 PFU ($p < 0.0052$, and $p < 0.094$, respectively). The addition of adjuvant IL-2 to the v. MCMHA/b-gal treatment group resulted in a 2 fold reduction in the number of pulmonary metastases in the group treated with v. MCMHA//B-gal primed splenocytes at 10^5 PFU.

Vaccination with a rVV expressing only the model TAA, was capable of priming a specific immune response which mediated tumor regression on adoptive transfer. Vaccination with a rVV containing both the costimulatory molecule, B7-1, and the model TAA, increased the therapeutic response by up to 150 fold. The increased therapeutic benefit mediated by v. MCB7-/b-gal primed lymphocytes may represent either or both a quantitative increase in the precursor frequency of TAA specific CTL in the adoptively transferred population, or a qualitative increase in the cytotoxic potential of the transferred cells mediated by B7/CD28 costimulatory signals. Infection with v. MCB7-1/b-gal would provide any MHC class I expressing cell the required elements necessary to mediate a CTL response, without the need for APCs or CD4+ T helper cells (Dohring, C. et al Int. J. Cancer 57:754-

759, 1994; Wu, Y. et al Curr. Biol. 4:499-505, 1994). Infection with v. MCB7-1/b-gal would also provide T cells with the capacity for autonomous stimulation leading to both a proliferative response to antigen and increased cytokine production (Dohring, C. et al ibid; Azuma, M. et al J. Exp. Med. 177:845-850, 1993). Notably, binding of B7/CD28 costimulates IL-2 mRNA accumulation (Linsley, P.S. et al J. Exp. Med. 173:721-730, 1991) important for T cells proliferative response, as well as IFN-γ, important in Th1 induction, as well as IL-4 important in Th2 induction (Walter, H. et al. Eur. Cytokine Netw. 5:13-21, 1994). In the absence of an antigen non-specific costimulatory signal delivered by interaction between B7-CD28, naive T cells exhibit suboptimal proliferation and decreased stability of mRNA for several cytokines important to the T cell proliferative and differentiation response (Linsley, P.S. et al Annu. Rev. Immunol. 11:191-212, 1993; Harding, F.A. et al J. Exp. Med. 177:1791-1796, 1993). Coculture of CD8+ T cells with B7+, but not B7- tumors results in potent cytotoxic response, and proliferation (Harding, F.A. et al J. Exp. Med. 177:1791-1796, 1993), which are inhibited by both anti-CD28 Fab fragments, and interestingly by anti-IL-2 antibodies. The results demonstrate that direct activation of CTL by rVV which provide appropriate costimulation and antigen specificity results in improved therapeutic response upon the adoptive immunotherapy of primed splenocytes to syngeneic tumor-bearing mice. The mechanism responsible for this response may be due to either or both a qualitative increase in antigen specific T cell precursors mediated by B7/CD28 (or CTLA-4) interaction, or B7/CD28 (or CTLA-4) mediated differentiation of T cell effector populations.

The adjuvant benefit received by adjuvant IL-2 alone upon the adoptive transfer of splenocytes in these studies confirms prior reports documenting direct IL-2 antitumor

responsiveness. The simplest explanation for the therapeutic response seen with IL-2 alone is that is represents a lymphokine activated killer cell (LAK) response mediating non-specific cytotoxicity. However, IL-2 treatment was therapeutic only in the mice bearing CT26.C25 tumors, which expressed the model TAA. Therefore, a more likely explanation for results seen with IL-2 alone and as an adjuvant, is an IL-2 mediated proliferation of antigen-specific CTL.

Example 23

Active Immunotherapy of Established Pulmonary Metastases with v. MCB7-1/β-gal

Treatment of established disease remains the ultimate goal of immunotherapy. If lack of costimulation is a principal means through which tumors evade immune surveillance in this model system, then vaccination with a v. MCB7-1/b-gal should generate an active and specific therapeutic immune response in animals bearing established tumor. To test this hypothesis, tumor-bearing mice were immunized with a single injection of rVVs, with designated groups receiving adjuvant treatment with IL-2 as outlined above. In addition to v. MCB7-1/b-gal and v. MCMHA/b-gal, v. MCB7-1/NP was used in these studies. The v. MCB7-1/NP co-expresses the B7-1 protein with an irrelevant protein (influenza nucleoprotein, NP) not expressed on the CT26.C25, and allows for assessment of the role of B7-1 expression alone. No significant tumor regression was seen following rVV vaccination in mice bearing CT26.WT tumors.

In mice bearing CT26.C25 tumors, immunization with v. MCB7-1/b-gal mediated a significant reduction in the number of metastases ($p < 0.006$) (Fig. 17.). The high therapeutic effect precluded evaluation of the effect of adjuvant IL-2. In the same experiment, mice immunized with v. MCMHA/b-gal mediated no significant tumor regression ($p < 0.36$). In four separate experiments a

significant decrease in the number of pulmonary metastases for mice immunized with v. MCMHA/b-gal at 10^7 PFU was seen only once ($p < 0.009$). IL-2 administration did not significantly improve upon tumor regression in v. MCMHA/b-gal treated mice. In the same and subsequent experiments, v. MCB7-1/NP had no effect on tumor regression. Overall, mice immunized with v. MCB7-1/b-gal showed a 170 fold and 117 fold greater reduction in the number of pulmonary metastases than mice in the v. MCMHA/b-gal and v. MCB7-1/NP immunization groups, respectively. Exogenous IL-2 alone did not mediate further tumor regression. This data implies that expression of the TAA and the costimulatory molecule are both necessary and sufficient to effect maximal regression of tumor in this active treatment model and suggests a role for B7-1 in both the induction and effector phase of the CTL response. This conclusion is further supported by the finding that a vaccination vector combining b-gal with an irrelevant protein did not reliably reduce tumor burden. (Note in one of four experiments, a significant reduction was observed, although to a much lesser degree than that seen in the v. MCB7-1/b-gal immunization group.) The presence of B7-1 alone was insufficient to effect any significant regression, which is consistent with reports that the mere expression of B7-1 on tissue without a new or foreign antigen does not result in tissue specific autoimmunity (Guerder, S. et al Immunity 1:155-166, 1994; Harlan, D.M. et al Proc. Nat'l Acad. Sci. U.S.A. 91:3137-3141, 1994).

The results from four separate active immunotherapy studies of mice immunized with 10^7 PFUs of rVV were normalized to the HBSS group for each individual experiment, and the data pooled and analyzed as percent residual disease (data not shown). Immunization with v. MCB7-1/b-gal group showed an almost 20 fold reduction in the tumor burden compared to HBSS control, with only 5.2% of tumor remaining. Adjuvant IL-2 treatment did not

improve upon this result, though a trend was seen in this group and the v. MCMHA/b-gal group. Immunization with v. MCMHA/b-gal mediated a nearly 2 fold reduction in the number of metastases with 40-55% of tumor burden remaining, while the v. MCB7-1/NP mediated no meaningful reduction with 83-93% of tumor burden remaining. This cumulative data confirms the finding seen in the individual active immunotherapy studies.

In order to evaluate whether active immunotherapy results with v. MCB7-1/b-gal were limited to the treatment of small tumor burdens, the same active treatment experiment in mice bearing six day metastases as repeated. A six day tumor burden is macroscopically visible with individual metastases measuring up to 2.5 millimeters. Again, no significant tumor regression was seen following rVV vaccination in mice bearing CT26.WT tumors. Figure 18 demonstrates that in the six day model, a single immunization with v. MCB7-1/b-gal mediated a significant reduction in the number of pulmonary metastases in mice bearing CT26.25 tumors ($p < 0.008$). Mice immunized with either v. MCMHA/b-gal or v. MCB7-1/NP had no significant reduction in the number of pulmonary metastases ($p < 0.12$, and $p < 0.22$, respectively). Again, adjuvant IL-2 did not improve this result. It is concluded therefore, that a rVV co-expressing both the costimulatory molecule, B7-1, and the TAA, b-gal, are required to mediate regression of both large and small tumor burdens in this model system. Consistent with the previous data disclosed herein and in Wang et al J. Immunol. 154(9): 4685-4692, 1995, immunization with a rVV containing only the immunodominant TAA, b-gal, was capable of inducing a cytotoxic immune response resulting in a reduction in the number of pulmonary metastases when small tumor burdens are present, but has no efficacy against a larger tumor burden. Immunization with a rVV expressing the costimulatory molecule alone without the TAA, i.e., v. MCB7-1/NP, was

unable to prime an immune response and mediate tumor regression in either situation.

A reduction in tumor burden is frequently demonstrated for several adjuvant interventions, but rarely translates into a therapeutic benefit in terms of prolonged survival. In a parallel experiment to the three day active treatment protocol described above, ten mice per group were followed daily for survival and events were recorded as either deaths, or severely moribund condition requiring euthanasia. In this study,all mice inoculated with CT26.WT were dead by day 43 with 50% dead in all groups by day 32.

CT26.C25 tumor bearing mice demonstrated varying degrees of survival among vaccination groups (Figure 19). Mice receiving HBSS, IL-2 alone, and v. MCB7-1/NP alone (with or without adjuvant IL-2), were dead by day 39. Mice vaccinated with v. MCMHA/b-gal showed a significant prolongation of survival with 50% of mice alive at day 27, and the last mouse dying at day # 46 ($p < 0.0002$) Adjuvant IL-2 did not statistically improve upon this result. In the v. MCB7-1/b-gal vaccinated mice 50 % were alive at 70 days, and 30% continue to be long term survivors at over 100 days ($p < 0.0001$). Adjuvant IL-2 therapy for this group added no statistically significant benefit to survival, though a trend was suggested with 50% still alive at day # 88, and 40% alive at 100 days follow-up.

The regression of pulmonary metastases seen following immunization with the v. MCB7-1/ b-gal thus translated into prolonged survival for most animals treated. Two mice who were long-term survivors in the v. MCB7-1/b-gal treated group, subsequently died and their lungs were subjected to whole organ X-gal staining. One of the mice showed no evidence of X-gal staining, despite bulky tumor metastases. The second mouse showed moderate positivity for X-gal staining, with some metastases exhibiting no

staining. Although the outgrowth of b-gal negative tumors as the cause of death in these long term survivors can not be concluded, it is a likely possibility.

Example 24

Active Immunotherapy of Established Pulmonary Metastases with v. B7-2/β-gal and v. MCB7-1/B7-2/β-gal

As stated earlier, optimal T cell activation require two signals, once delivered by the MHC/TCR interaction and a second from the B7-1/CD28 or CTLA-4 interaction. It was evaluated whether a rVV coexpressing B7-2 (v. MCB7-2/β-gal), or both B7-1 and B7-2 (v. MCB7-1/B7-2/β-gal), with a TAA would be a more potent vaccine to stimulate TAA specific CTL response.

Mice were initially inoculated with 5 x 10^5 tumor cells of CT26.WT or CT26.C25. Mice bearing three day tumors were then immunized with a single injection of rVV, with designated groups receiving adjuvant treatment with IL-2 as outlined above. In addition to the rVV described, an additional rVV was constructed v. MHA/B7-2/b-gal, which contained the MHA gene in the Hind III region of the VV genome, and both B7-2 and the E. coli lac Z gene in the TK region. This was created to control for the presence of the B7-1 gene in the v. MCB7-1/B7-2/β-gal construct (Fig. 20.). No significant tumor regression was seen following rVV vaccination in mice bearing CT26.WT tumors.

As seen in previous active immunotherapy experiments with mice bearing three-day CT26.C25 tumors, vaccination with v. MCB7-1/β-gal resulted in a significant reduction in the number of pulmonary metastases ($p < 0.0014$), while v. MCMHA/β-gal mediated only a minimal reduction ($p < 0.054$), and v. B7-1/NP mediated no reduction ($p < 0.13$). Vaccination with both v. MCB7-2/β-gal and v. MCB7-1/B7-2/β-gal resulted in a significant reduction in the number of pulmonary metastases ($p < 0.0018$ and $p < 0.0018$, respectively). Similarly, treatment with v. MCMHA/B7-2/β-gal also mediated significant reduction in the number of